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Fluorescent Biosensors for Real-time Tracking of Post-translational Modification Dynamics

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Abstract

Dynamic post-translational modifications (PTMs) regulate and diversify protein properties and cellular behaviors. Real-time monitoring of these modifications has been made possible with biosensors based on fluorescent proteins (FPs) and fluorescence resonance energy transfer (FRET), which can provide spatiotemporal information of PTMs with little perturbation to the cellular environment. In this review, we highlight available fluorescent biosensors applicable to detect PTMs in living cells and how they have shed light on biological questions that have been difficult to address otherwise. In addition, we also provide discussions about various engineering strategies for overcoming potential challenges associated with the development and application of such biosensors.

Introduction

Protein post-translational modifications (PTMs) are the chemical modifications that occur on specific amino acid(s) of a protein after it is being translated, including covalent attachment of phosphate, alkyl groups, carbohydrates, polypeptides, and others. A few well-known examples of PTMs are phosphorylation, methylation, glycosylation and ubiquitination. These modifications serve the important purpose of increasing a protein's functional diversity by altering its basic physical and chemical properties such as its structure, activity, stability, cellular localization and interaction profile [1]. In fact, the very same protein can carry out distinct functions depending on its specific modifications [2]. As a result, a limited number of genes can be regulated in such a way to generate seemingly far more complex cellular behaviors.

Much of our knowledge about PTMs has come from studies employing analytical methods such as *in vitro* biochemical assays, immunofluorescence microscopy and mass spectrometry [3,4]. While these conventional methods have provided important information regarding the identifications and regulations of various PTMs, in general, they lack the ability to track real-time dynamics of PTMs in single living cells. However, it is believed that such dynamic regulation is essential to their biological functions [5]. Therefore, it is important to develop

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methods and tools that offer such capability. In recent years, direct visualization of a variety of PTM events in living cells has been made possible by the development of fluorescent biosensors based on fluorescent proteins (FPs) and fluorescence resonance energy transfer (FRET). These genetically encoded biosensors have enabled us to better understand the dynamic regulation of protein behaviors by various PTMs in their native context, the living cell.

FRET-based biosensors for post-translational modification dynamics

FRET is the nonradiative transfer of energy from an excited donor fluorophore to an acceptor fluorophore in its proximity [6]. For FRET to occur, the emission spectrum of the donor fluorophore should overlap with the excitation spectrum of the acceptor fluorophore and the two fluorophores must be in close proximity (<10 nm). Importantly, FRET efficiency is highly sensitive to the distance and/or orientation between the donor and acceptor fluorophores [7]. For FRET-based biosensors discussed here, two FPs are chosen so that the emission spectrum of the donor FP sufficiently overlaps with the excitation spectrum of the acceptor FP, for example cyan FP (CFP) and yellow FP (YFP). Furthermore, all these biosensors have been designed to achieve a FRET change via either a conformational change (for intramolecular or unimolecular biosensors) or a distance change (for intermolecular or bimolecular biosensors) [7,8•]. The change in FRET is then used as a readout for the change in PTM dynamics. While there are many types of PTMs, currently available biosensors (Table 1) are limited to only a few, including phosphorylation/dephosphorylation, methylation, ubiquitination, and glycosylation.

Protein phosphorylation is the covalent attachment of a phosphate group, for example at the serine amino acid residue, to a target protein. This modification is carried out by a specialized family of enzymes known as protein kinases, which are involved in every aspect of signal transduction. Therefore, understanding of the spatiotemporal regulation of protein kinases in living cells holds the key to deciphering the logic and language of signal transduction. Toward this end, FRET-based kinase activity reporters for various serine/threonine and tyrosine kinases have been developed for monitoring their phosphorylation dynamics in living cells [9•-14••]. The modular design of a typical kinase activity reporter consists of two FPs (Figure 1a), a target substrate for the specific kinase of interest and a phopho-amino acid binding domain that serves as an interaction partner of the phopho-substrate to induce a conformational change upon substrate phosphorylation. On the other hand, dephosphorylartion is carried out by another family of enzymes known as protein phosphatases that often work alongside protein kinases to control the phosphorylation state of their substrates in a cellular context. Recently, a FRET-based biosensor has been developed to specifically track the activity of the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, calcineurin, in living cells [15]. This reporter design utilizes a phosphatase activity-dependent molecular switch based on a specific substrate of calcineurin, NFAT, sandwiched between CFP and YFP, and should be generally applicable to other protein phosphatases.

Protein methylation is the covalent attachment of a methyl group, for example at the lysine amino acid residue of a histone protein, to a target protein. In particular, methylation of the N-terminal tails of histones is involved in transcriptional regulation and can be quite dynamic. To monitor the dynamics of histone methylation in living cells, a couple of FRET-based fluorescent reporters have been constructed using residues 1−13 (K9 methylation) or 24−35 (K27 methylation) of histone H3 as the substrate and HP1 chromodomain or Polycomb chromodomain as the methylation recognition domain for the K9 and K27 methylation, respectively [16]. These reporters take advantage of the same modular design employed by the FRET-based kinase activity reporters since, like phosphorylation, methylation occurs on specific substrates and is recognized by specific binding domains.

O-GlcNAcylation, a type of protein glycosylation by which the β-*N*-acetylglucosamine (O-GlcNAc) attaches to the serine/threonine residue of a target protein, is a dynamic modification that plays an important role in signal transduction. To visualize the O-GlcNAcylation dynamics in living cells, a FRET-based biosensor has been developed using a substrate for O-GlcNAc transferase derived from casein kinase II and an O-GlcNAc-binding domain, GafD [17]. On the other hand, O-GlcNAcylation has been shown to compete for the same modification sites with protein kinases and thereby negatively regulate kinase signaling [18]. As such, biosensors like this should provide unique opportunities to dissect the dynamic interplay between O-GlcNAcylation and phosphorylation in living cells.

Additionally, a biosensor for ubiquitination, a modification that attaches a small protein called ubiquitin to a target protein, has been developed based on bioluminescence resonance energy transfer (BRET), a process similar to FRET but using bioluminescence instead [19].

Biological insights

Importantly, applications of these fluorescent biosensors have enabled us to gain biological insights into the spatiotemporal regulation of various PTMs in single living cells. Highlighted in the following paragraphs are a few recent examples.

In cardiac myocytes, cAMP/PKA signaling via β-adrenergic receptors (β-ARs) represents a key mechanism for controlling cardiac contraction under neurohormonal stimulation. Upon activation by cAMP, PKA phosphorylation of multiple proteins targets, including phospholamban and activated receptors, lead to enhancement of myocyte contraction as well as desensitization of activated β-ARs [20,21]. Given the many protein targets involved in this process and their distinct functions as well as subcellular confinement, it is important to understand how signaling specificity from PKA to its respective substrates is achieved. Using A-kinase activity reporter (AKAR), which can dynamically monitor the level of PKA substrate phosphorylation by providing a corresponding FRET signal [22], it was found that in both wild-type and β_2-AR-KO cardiac myocytes, activation of β-ARs lead to sustained PKA activation that results in an increase in phospholamban phosphorylation and thus enhanced myocyte contraction [23••]. In contrast, transient cAMP accumulation has been reported under similar conditions [24,25]. Furthermore, selective stimulation of β_2-AR induces transient PKA activation that is sufficient to phosphorylate activated receptors on plasma membrane but not phospholamban, which is on the sarcoplasmic reticulum, suggesting spatiotemporal control of PKA activation can lead to differential substrate phosphorylation (Figure 1b). Together, these findings have provided answers to two important outstanding questions with regard to β-AR signaling in cardiac myocytes: 1. Although it is well-accepted that β-AR-stimulated cAMP signaling is responsible for the enhancement of myocyte contraction, there exists an apparent lack of temporal correlation between the transient cAMP accumulation and the more sustained contraction responses; 2. While stimulation of β_2-AR induces significant cellular cAMP accumulation, it has little effect on the myocyte contraction responses.

Aurora B kinase is a key mitotic regulator that plays multiple roles during cell division, including ensuring proper chromosome attachment to the mitotic spindle [26]. However, little has been known about the connection between its sptiotemporal regulation and its diverse functional roles. In a recent study, aurora B signaling patterns in living cells has been quantitatively analyzed using a FRET-based aurora B kinase activity reporter [14••]. It was found that reporters targeted to different subcellular locations showed site-dependent aurora B activity kinetics during anaphase. In addition, the kinetics of the chromosome-targeted reporters was similar to that of chromosome segregation, suggesting a correlation between aurora B activity and chromosome position. Indeed, further analysis of reporter signal with regard to centromere position at time points early in anaphase revealed a striking spatial phosphorylation gradient with the highest phosphorylation level at the spindle midzone. This

phosphorylation gradient due to midzone activation of aurora B has been proposed to provide important spatial information for mitotic events.

Ubiquitination is the PTM that enables rapid protein degradation. It is a highly dynamic and regulated process that also plays a prominent role in a variety of other cellular signaling processes [27]. Thus, being able to visualize the real-time dynamics of ubiquitination should lead to better understanding of how ubiquitination regulates various cellular functions. To study the ubiquitination dynamics of β-arrestin, an important regulator of G-protein coupled receptor (GPCR) signaling, a BRET-based ubiquitination biosensor has been developed. This bimolecular biosensor consists of a luciferasetethered ubiquitin substrate, β-arrestin, and a GFP-tethered ubiquitin, where luciferase and GFP serve as energy donor and energy acceptor, respectively [19]. Using this biosensor, it was shown in living cells that activation of β_2-AR produces a transient β-arrestin ubiquitination, whereas selective activation of another GPCR, V2-vasopressin receptor, leads to a sustained β-arrestin ubiquitination. In addition, parallel tracking of β-arrestin recruitment to the activated GPCRs using a similar BRET-based approach revealed a tight correlation between the β-arrestin ubiquitination and its recruitment to the activated receptors. Together, these findings suggest that the distinct kinetic changes of β-arrestin ubiquitination with regard to GPCR signaling are indeed determined by its interaction profiles with the receptors [28].

Discussions

Most PTMs involved in cellular signaling are very dynamic and the signals are often encoded in relative changes in the PTM state of their target substrates. Therefore, it is imperative that PTM biosensors have sufficient dynamic range to track the physiologically relevant changes of PTM dynamics which can be relatively small. Furthermore, improved dynamic range should make high-throughput monitoring of PTM dynamics in living cells possible and enables efficient screening for specific small molecule regulators of PTMs [29]. In turn, these potential regulators should help elucidate the molecular mechanisms underlying the dynamic regulation of various PTMs. Moreover, these regulators can be potentially useful for correcting dysregulated PTM signaling. Several practical strategies have been successfully applied to the improvement of various existing biosensors [22,30]. Among these, the most successful one to date is the change of FPs used as the FRET pair in the biosensor, including using FPs with improved spectral properties for FRET and circular permutated FPs, since FRET efficiency depends on the spectral properties of the donor and acceptor fluorophores and is highly sensitive to the relative orientation between them.

Not only PTMs are dynamic, often they are also associated with spatially defined signaling patterns. To be able to visualize such spatially regulated signaling, genetically encoded biosensors can be targeted to subcellular regions of interest via specific amino acid targeting sequences. For example, while the chromosome-targeted aurora B kinase activity reporters revealed a midzone phosphorylation gradient during anaphase, the untargeted version of the reporter failed to do so [14••]. In another example, targeting C-kinase activity reporter (CKAR) to the plasma membrane where protein kinase C (PKC) is activated, revealed oscillatory phosphorylation in HeLa cells in response to histamine [31]. In contrast, untargeted CKAR not only did not detect phosphorylation oscillation, it actually did not show discernable signal at all under the same condition.

In addition, PTMs do not happen in isolation in a cellular context. Rather, a single signaling process may involve multiple PTMs [32]. Furthermore, multiple PTMs, for example, O-GlcNAcylation and phosphorylation, can compete for the same modification site on a particular protein substrate [18,33]. Thus, it is advantageous to study the dynamics of related PTMs in parallel, allowing for precise spatiotemporal correlation of these PTMs in the same cellular

context. Toward this end, recent advances in FP engineering have afforded us with a rainbow of FPs [34•], making it possible to employ spectrally distinct FRET pairs for parallel tracking of multiple events in a single living cell [35•]. A recent progress in multiparameter imaging is the development of a single-excitation dual-FRET method that allows precise parallel tracking of fast signal dynamics in cells undergoing significant morphological changes [36].

So far, only a limited number of PTMs can be dynamically tracked with fluorescent biosensors. This is, in part, due to the lack of specific information about many PTMs, including substrate specificity, localization pattern, interaction profile and regulation. Such information can be obtained from traditional methods such as *in vitro* biochemical assays, immunofluorescence microscopy and mass spectrometry. However, to accelerate the development process, alternative methods and tools are needed to yield information more relevant to the development of biosensors for particular PTMs. For example, FP-based fragment complementation assay can provide information about protein-protein interactions in living cells [37], thereby facilitating simultaneous selection of a specific target substrate and a corresponding recognition domain: two important modular components in a FRET-based biosensor for detecting PTM dynamics. Similarly, FRET- or BRET-based assays can also be developed to provide such information. In a recent study, selective recognition of acetylated histones by bromodomain proteins in living cells has been determined employing a FRET-based flow cytometry method [38]. Specifically, cells expressing different combinations of FP-tagged (CFP and YFP, respectively) bromodomain and histone proteins were analyzed for FRET signals between CFP and YFP in order to determine the specific interactions. This may be an important step toward the development of a biosensor for dynamic histone acetylation.

Conclusion

In conclusion, FRET-based fluorescent biosensors have served as important tools in real-time tracking of spatiotemporal dynamics of PTMs in living cells. These genetically encoded biosensors offer precise molecular targeting, as well as high spatiotemporal resolution in living cells. In addition, the very same advantages can be extended to more complex living systems such as tissues and whole organisms. Therefore, continuing developments of biosensor for other PTMs along with better engineering strategies to allow for improved dynamic range and simultaneous monitoring should lead to better understanding of the complex molecular mechanisms underlying these dynamic signaling processes in living systems.

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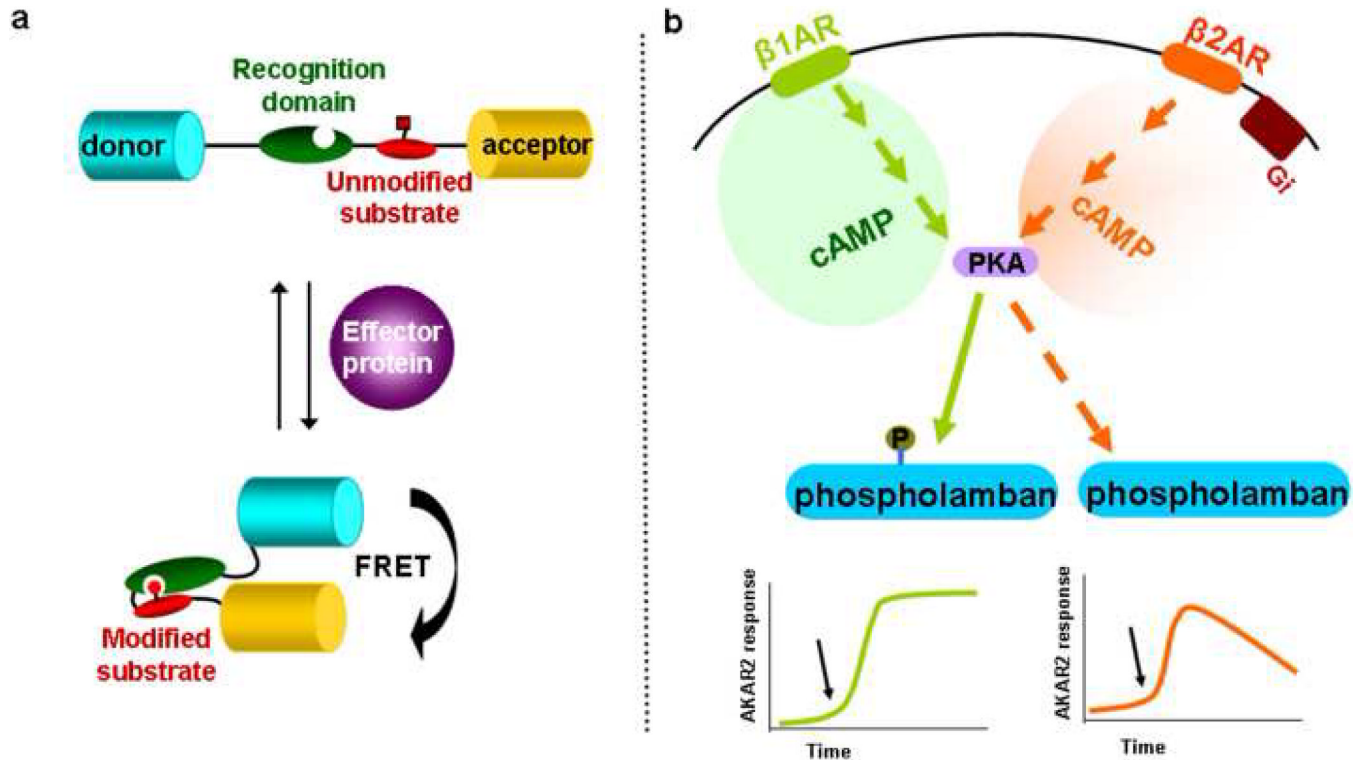

Figure 1. General biosensor design and its application in living cells

(a) A schematic construction of unimolecular FRET-based biosensors for post-translational modification. The modular components are a FRET-donor and acceptor pair, a substrate domain that is subject to modification and a recognition domain that can recognize the particular post-translational modification. PTM dynamics can be measured by changes in FRET. (b) Temporal dynamics of PKA phosphorylation observed by PKA activity reporter, AKAR2. Black arrow indicates the activation of respective receptors.

Table 1

A list of biosensors that detect dynamics of different PTMs.

Currently available biosensors to detect dynamics of different PTMs		
	Biosensor name	**References**
Glycosylation		
β-O-GlcNAcylation		17
Methylation		
Histone K9/K27 methylation	K9/K27 reporter	16
Phosphorylation (kinase/phosphatase activity reporters)		
Protein Kinase A (PKA)	AKAR	9•
Protein Kinase B (PKB/Akt)	BKAR, AktAR	9•,10
Protein Kinase C (PKC)	CKAR	9•,27
Protein Kinase D (PKD)	DKAR	9•
Extracellular Signal Regulated Kinase (ERK)	ERKUS, EKAR	9•,12
Crk II adaptor protein	Picchu	9•
Src, EGFR		9•
Src		11
Aurora B Kinase		14••
Histone H3 phosphorylation		13
Phosphorylation	Phocus	9•
Calcineurin activity reporter	CaNAR	15
Ubiquitination		
β-Arrestin ubiquitination		19